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DIFFERENTIATION OF HEMOLYTIC STREPTOCOCCI FROM MILK AND
FROM HUMAN LESIONS

DISSERTATION

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The work presented in this paper was undertaken primarily to study the characters which differentiate hemolytic streptococci which are pathogenic for man from non-pathogenic hemolytic streptococci occurring in milk. Several papers in which the finding of hemolytic streptococci in milk is reported have appeared within the last few years. These streptococci are generally considered to be non-pathogenic, except in a few cases in which hemolytic streptococci have been found in milk during epidemics of septic sore throat and were thought to be the etiologic agent in the disease.

Streptococci which produce a clear zone around the colony on a blood agar plate are usually regarded as hemolytic. There are wide variations among streptococci in their ability to produce zones of hemolysis. In fact, most streptococci and some pneumococci cause a slight clearing of blood agar around the colony. Smith and Brown (1) have studied the hemolytic zones made by streptococci on standardized blood agar plates and have classified them according to the appearance of the zone of hemolysis. It was found that hemolytic streptococci of the typical human pathogenic type gave a perfectly well-defined zone of hemolysis two to four millimeters in diameter. These streptococci were recognizable on blood agar plates and were found to be a group with definite pathogenic properties. This group is designated by Smith and Brown as

the Beta type.

It has been observed by several investigators, however, that streptococci which seem to have no pathogenic properties, but which produce, on blood agar plates, zones of hemolysis closely resembling the Beta type, frequently occur in milk. Davis (2) found streptococci of this kind in both raw and pasteurized milk but more commonly in the former. Most of them were non-virulent for rabbits. Brown (3), in a recent paper, points out some of the characteristics which differentiate milk strains from human pathogenic strains.

Preliminary examination of pasteurized milk in Baltimore showed that streptococci of the Beta type of hemolysis were present in the milk, in some cases in large numbers. A survey was made of the milk of a number of dairies to determine how generally these streptococci occurred. The bottled milk was bought from the dealers and plated on standard blood agar plates. Plain agar was used in the preparation of the blood agar. This was tubed in 12 cc. portions and to the melted and cooled agar, .5 cc. of whole rabbit's blood was added. Dilutions of the milk were made and plates were poured. The plates were incubated for forty-eight hours at 37° C. The number of colonies showing hemolysis of the Beta type were counted and recorded. The numbers of these hemolytic streptococci in the milk of a given dairy varied widely from day to day. Also the proportion of hemolytic streptococci to other organisms varied widely. Table I represents typical counts

Table I.

THE OCCURRENCE OF HEMOLYTIC STREPTOCOCCI IN MILK.

DAIRY	HEMOLYTIC STREPTOCOCCI PER GENT OF HEMOLYTIC:		
	BACTERIA PER CC.:	PER CC.	STREPTOCOCCI
A	45 000	3 800	2.0
B	22 000	320	1.4
C	157 000	340	0.4
D	22 000	1000	0.0
*E	8 300	500	0.4
F	66 000	525	0.9
G	15 400	400	0.4
B	15 000	190	1.3
E	12 300	125	1.0
H	44 300	8 500	19.1
*E	6 950	235	0.2
D	26 000	300	0.0
E	22 500	100	0.4
I	55 600	1000	0.0

of the milk of several dairies.

The similarity of these pathogenic and non-pathogenic streptococci on blood agar plates and the frequency with which the latter occur in milk render the study of characteristics which differentiate them of importance.

HISTORICAL.

A great deal of work has been done on the differentiation of streptococci and many characters are said to have differential value. A complete review of the voluminous literature on differentiation of streptococci will not be attempted, as several very complete bibliographies of the streptococci have been published recently. It is purposed here to name the more common methods of differentiation and to review briefly some of the studies made of each.

MORPHOLOGY: The earliest attempts at classification of streptococci were based on morphology and particularly on chain formation. v. Lingelsheim (4) named the long chain forms S. longus and the short chain forms S. brevis. Such a classification has very little value since the length of chain is largely dependent upon the medium in which the organism is grown. It has often been observed, however, that long chain forms are more likely to be pathogenic than are short chain forms. It seems to be the generally accepted opinion, however, that no particular classificatory value should be attached to chain formation. The form of the individual cell is likewise

somewhat variable. Oval and cylindrical forms often occur in strains which are usually spherical. The usual text book description of *Streptococcus lacticus* characterizes this group as being generally more oval or lanceolate than the *Streptococcus pyogenes*. Morphological characteristics may be of value in confirming one's judgment based on more stable characteristics.

FERMENTATION OF CARBOHYDRATES: Undoubtedly the most extensively studied characteristic of the streptococci is their biochemical activity in media containing carbohydrates. Gordon (5) made the first extensive study of the fermentation of carbohydrates, and related substances, by streptococci. He studied ten cultures from different sources with thirty-five different test substances. From these substances he selected two disaccharides, lactose and saccharose; one trisaccharide, raffinose; one polysaccharide, inulin; two glucosides, salicin and coniferin; and one alcohol, mannite. He applied the fermentation of these test substances, with the reduction of neutral red and the curdling of milk, to 300 cultures from normal saliva and to 100 from the air. The test substances divided these streptococci into forty-eight groups. Following this work a great many studies of streptococci from various sources have been made, using the Gordon test substances. Other characters have been added and attempts made to correlate them with fermentation.

Houston (6) studied 300 cultures from human feces,

173 from milk, and 100 from cow dung. In addition to the fermentation of the Gordon test substances, he observed the appearance of broth cultures, liquefaction of gelatin, reduction of nitrates, and pathogenicity for mice.

Andrews and Horder (7) studied 1300 strains and made the fermentation of Gordon's test media, growth at 30° C., the formation of chains, and pathogenicity for mice the basis of a classification of streptococci in which six species were recognized. This classification, however, has not gained general acceptance.

Savage (8) used carbohydrate fermentation in the study of streptococci of milk from normal and gargety cows and from sore throats. Growth at low temperature, length of chains, and mouse pathogenicity were also emphasized. Many groups were found with the use of these characters.

In this country, Winslow and Palmer (9) used four carbohydrates, namely, dextrose, lactose, raffinose, and mannite in the study of bovine and equine fecal streptococci. They introduced quantitative acid production in the study of streptococci and believed that the amount of acid produced with these carbohydrates characterized definite groups. In a later paper, Winslow (10) advocates the use of statistical or biometrical studies of fermentation.

Stowell, Hilliard, and Schlesinger (11) tried to correlate a number of characters, such as morphology, character of growth, hemolysis, and resistance to gentian violet

with fermentation but failed to find any constant correlation. The production of acid at low temperature seemed to be of value in separating the milk strains from those found in throats.

Fuller and Armstrong (12) used quantitative acid production in studying the fecal streptococci of the horse, the cow, and man. The percentage of the various fermentation groups found in the different feces do not agree closely with those of Winslow and Palmer (9).

Throughout the literature bearing on the fermentation reactions of the streptococci, widely varying results are obtained by different workers. Winslow (10) and Broadhurst (13) show that differences in media, indicators, and other technical factors may be the cause of these discrepancies. Thro (14) also offered a list of technical errors to explain the varying results. Holman (15) points out other errors and calls special attention to the difficulty of isolating streptococci in pure culture. He also proposes a standard medium for testing fermentation. Hemolysis and the fermentation of four carbohydrates are made the basis of his classification of streptococci.

Kligler (16) studied the correlation of fermentation with hemolysis and agglutination. He concluded that fermentation represents relationships because of its correlation with agglutination, and that division on the basis of hemolysis is not warranted. Hopkins and Lang (17) also discard hemolysis

in favor of fermentation reactions. Titration of acidity and coagulation of milk are not considered of much value.

Most investigators find that in using fermentation tests in the classification of streptococci the majority of strains fall into a few groups, but besides these there are a large number of groups represented by a very few strains. It is doubtful whether or not these small groups separated from the larger ones only by a positive or negative in a single test substance are sufficiently different to be separately classified. If such groups are recognized, their number increases geometrically with the number of test substances used. The tendency in the more recent work has been to use a small number of test substances and to correlate the results with other characters. The question of fermentation has been so generally studied that an investigation of any group of streptococci would seem incomplete without including their fermentation reactions.

HEMOLYSIS: Schottmuller (18) was the first to use hemolysis as a basis of classification. He described the appearance of S. longus pathogenes, S. mitior, S. mucosus, and the pneumococcus on blood agar plates. Human blood was used. Zangemeister (19) studied hemolysis with the blood of man, horse, rabbit, cow, hog, hen, and pigeon. Differences in hemolysis were noted but the blood of no species gave negative results when others were positive. The zone of hemolysis was also varied by the quantity of blood.

Hoessli (20) concluded that the hemolytic character of streptococci is far too variable to be used in differentiation. While Rolly (21) found hemolysis constant and satisfactory for classification. Saito (22) found hemolysis constant for the blood of a given species but somewhat different for the blood of different species. Jaffe (23) stated that hemolysis is unsuited for differentiation because it is an inconstant characteristic.

Kuhn (24) shows that sugar in blood media reduces hemolysis. Ruediger (25) states that S. lacticus can be differentiated from S. pyogenes by the fact that the former produces no zone of hemolysis on blood agar. M'Leod (26) concludes that hemolysis on blood agar plates is unreliable and that the minimum hemolytic dose should be determined instead.

Hopkins and Lang (17) find that the majority of streptococci produce some hemolysis and that it is more pronounced in virulent types, but the difference is only quantitative and is not suitable for classification.

Smith and Brown (1) described two types of hemolysis produced by streptococci on blood agar plates. The appearance of the zones was carefully described, including the appearance under the microscope.

Holman (27) makes hemolysis the primary basis for a classification of streptococci, the groups are further sub-

divided by the use of fermentation tests. Smillie (28) confirms the observations of Smith and Brown (1) and emphasizes the fact that different types of hemolysis are produced with different blood.

From the literature it appears that differences in hemolysis produced on blood agar plates by the same organisms may be caused by the use of different kinds of blood, different quantities of blood and agar, the presence of carbohydrates, and other factors. The necessity of standard blood agar plates is discussed by Becker (29). He shows that the reaction of the medium and the crowding of colonies, in addition to some of the factors mentioned above, influence the zone of hemolysis.

The whole question of the use of blood agar is completely reviewed in a monograph by Brown (30). He proposes a standard blood agar plate and gives exact directions for making and seeding blood agar plates which will bring out the different types of hemolysis described by Smith and Brown (1). It seems now to be quite generally conceded that hemolysis is a constant characteristic when the organism is tested under constant conditions.

PATHOGENICITY: The pathogenicity of streptococci for laboratory animals has not been considered by itself as a basis for classification, but it is very desirable that characters which correlate with pathogenicity be found. In searching for characters which distinguish pathogenic strains, various direct criteria of pathogenicity and virulence have been proposed.

Frankel (31) found strains of S. mitis which were pathogenic for mice. Andrews and Horder (7) found that some of their strains of S. pyogenes and S. anginosus were pathogenic for mice, while S. salivarius and S. fecalis were non-pathogenic for mice. Ruediger (32) found S. pyogenes from scarlatinal and normal throats to be pathogenic for rabbits, while S. viridans was not. Horder (33) found cases of infective endocarditis due to streptococci which were non-pathogenic for mice. Sachs (34) states that pathogenicity for animals and pathogenicity for man do not run parallel. Bahr (35) found that S. lacticus was non-pathogenic for mice and rabbits. Saito (22) made the same observation, while Heineman (36) claimed to have raised the virulence of S. lacticus to the point of killing rabbits by successive passage through a number of rabbits.

A large number of investigators have found streptococci pathogenic for animals to be the etiological agent in milk borne sore throat epidemics. Davis and Rosenow (37) found such streptococci pathogenic for rabbits, guinea pigs, and mice. Stokes and Hachtel (38) found them pathogenic for mice. North, White, and Avery (39); Smith and Brown (1); Capps and Davis (40) found them pathogenic for rabbits. Smillie (28) found positive results with both mice and rabbits.

Davis (2) found hemolytic streptococci in milk which were generally non-virulent for rabbits. He expressed the belief that pathogenicity of hemolytic streptococci for rab-

bits ran parallel with pathogenicity for man.

Holman (15) has shown quite conclusively that animal experimentation with streptococci is subject to many errors on account of the normal streptococcus flora of the animal body. Stress should not be placed on the results of animal inoculation. In the work which has been done the methods have been far too variable to be compared. Different animals have been used, different amounts of inoculum, different sites of inoculation, and other factors which would render results incomparable. No standard test of virulence or pathogenicity has been established. Mice and rabbits have been used more than other animals.

SEROLOGICAL REACTIONS: Attempts to classify streptococci by their serological reactions have not met with great success. The specific agglutination of streptococci is subject to great difficulty because of the spontaneous agglutination which takes place in broth cultures of streptococci.

Davis (41) used anaphylactic reactions in guinea pigs to show relationships among streptococci. He believed that the results obtained indicated relationships between groups.

Kligler (16), as already stated, used agglutination and its correlation with fermentation reactions as a basis of classification of streptococci. He found no correlation between agglutination and hemolysis.

Kruzweide and Valentine (42) found no relationship

among streptococcus viridans by agglutination tests.

Kinsella and Swift (43) attempted to classify hemolytic streptococci by means of complement fixation reactions. They conclude from their results that the hemolytic streptococci are a homogeneous group. They abandoned attempts to use agglutination because of spontaneous agglutination among their cultures.

Howell (44) also studied complement fixation with streptococci. She found no correlation between this reaction and hemolysis or carbohydrate fermentation. She concluded that a classification based on complement fixation was not justifiable.

Recently Havens (45) has divided hemolytic streptococci into groups based on agglutination. He reports that no difficulty was encountered due to spontaneous agglutination when a twelve to sixteen hour blood broth culture was used.

In general, however, much doubt has been expressed as to the value of serological reactions in grouping streptococci.

OTHER CHARACTERS: Ayers, Johnson, and Davis (46) called attention to the difference in hydrogen-ion concentration in broth cultures produced by pathogenic and non-pathogenic streptococci. They found that in dextrose-yeast-peptone medium the limiting hydrogen-ion concentration for pathogenic streptococci was from P_H 5.4 to 6.0 and for non-pathogenic from

P_H 4.5 to 4.9. Avery and Cullen (47) studied the same character, using strains from human and bovine sources. The final readings with the human strains were from P_H 5.0 to 5.3, while the bovine strains were from P_H 4.3 to 4.5.

The curdling of milk was included in some of the earlier work on the classification of streptococci and has since been included in the studies of a great many workers. Andrews and Horder (7) found that their S. equinus, S. mitis, and S. pyogenes do not curdle milk, while S. salivarius, S. anginosus, and S. fecalis do coagulate milk. It has generally been found that saprophytic streptococci, or streptococci of low virulence, coagulate milk while the more pathogenic ones do not. It is only in a general way, however, that this distinction holds true. Pathogenic streptococci are found which do coagulate milk and some saprophytic ones are found which do not. The procedure is a simple one and its value is sufficient to justify its inclusion in the study of any group. In a general way, the results correlate with the determination of limiting hydrogen-ion concentration, that is, those which coagulate milk are usually those which produce a high acidity in dextrose broth media.

Another test which, for a long time, has been applied to streptococci is the reduction of dyes. Neutral red has been principally used. Gordon (5) used this test along with his studies of fermentation. Sherman and Albus (48) were able to differentiate streptococci of the pyogenes type from those

of the lacticus type by their reduction of methylene blue, litmus, indigo carmine, and neutral red. Brown (3) was unable to distinguish sharply between bovine and human hemolytic streptococci by this method.

Sherman and Albus (48) also found that growth at 10° C. and 43° C. help to differentiate pyogenic from lactic streptococci.

Ayers, Johnson, and Davis (46), in a study of the effect of pasteurization temperature upon streptococci, found that the pathogenic streptococci were easily killed at 60° C. while the non-pathogenic streptococci survived higher temperatures.

METHODS.

From a study of the literature on differentiation and classification of streptococci it becomes apparent that no single character will suffice to distinguish a group, though by the use of a group of characters it may be possible to define a fairly homogeneous group. In this work a study of the hemolytic streptococci which occur in milk was made with a threefold purpose in mind. It was purposed, first, to learn the characteristics of these streptococci and to see whether or not they constitute a homogeneous group; second, to determine the characters which are most useful in differentiating them from hemolytic streptococci which are pathogenic for man; and third, to study the thermal death rate of this group and

of other hemolytic streptococci in milk. The following characters were selected for study:

1. Morphology.
2. Fermentation reactions.
3. Hemolysis.
4. Pathogenicity for mice.
5. Reduction of litmus and coagulation of milk.
6. Thermal death rate in milk at 60° C.

The milk strains used in this study were isolated from milk by plating on blood agar. Most of them were taken from bottled pasteurized milk, but some were from certified unpasteurized milk. The blood agar was prepared by adding citrated whole rabbit's blood to plain agar. One-half cc. of blood was added to each tube containing 12 cc. of the melted and cooled medium. The dilutions of milk were made and then mixed with the agar on the plate. The plates were incubated for twenty-four hours at 37° C.

Only those colonies which gave wide, clear, well-defined zones of hemolysis were selected. Colonies showing every degree of hemolysis from a barely perceptible halo to the wide, clear zones were found on most of the plates. Some colonies gave wide zones but the outer limits of the zones were not well defined. Such colonies when fished to broth and the broth tested with washed red cells gave no hemolysis. Only those which had the wide, clear-cut zones were found to respond to this test. These colonies correspond to Smith and Brown's Beta type. After a little experience it was found possible to pick these colonies with considerable accuracy.

The selected colonies were transferred to veal infusion broth. Smears were made from the broth cultures and examined for purity. Stock blood agar slants were made from the pure broth cultures. All tests were made upon transfers from these stock cultures.

For comparison with the milk strains, a number of cultures from pathological sources were selected. Most of these were obtained from the Hygienic Laboratory of the United States Public Health Service, Washington, D. C. These cultures had been isolated mainly from empyema cases in army camps. Others were obtained from other sources as shown in Table III.

MORPHOLOGY.

A study of the morphology of the milk strains was made soon after isolation. For this purpose they were grown in meat infusion broth and stained by Gram's method, after twenty-four hours growth. The human strains were grown in broth and examined in the same way. Tables II and III show the summary of the points observed. Examination was also made of the growth from blood agar and from milk. In general, it may be said that the organisms were more uniform in size and shape when grown in milk than when grown in the other media. In broth and especially on blood agar the organisms tended to develop irregular shapes and sizes. This was more marked in the milk strains than in the human strains.



Culture	Source	Cells	Predominating Groups
W 4	Certified Milk	Spherical	Short chains
W 5	Certified Milk	Spherical	Short chains
W 6	Certified Milk	Spherical	Short chains and pairs
W 7	Certified Milk	Lance oval	Long chains
W 8	Certified Milk	Spherical	Short chains
D 6	Standard Pasteurized Milk	Lance oval	Short chains and pairs
D 7	Standard Pasteurized Milk	Lance oval	Short chains and pairs
D 8	Standard Pasteurized Milk	Lance oval	Short chains and pairs
D 9	Standard Pasteurized Milk	Lance oval	Short chains and pairs
D 10	Standard Pasteurized Milk	Lance oval	Short chains and pairs
D 11	Standard Pasteurized Milk	Spherical	Short chains and pairs
D 12	Standard Pasteurized Milk	Oval	Short chains and pairs
U 1	Standard Pasteurized Milk	Oval	Short chains and pairs
U 2	Standard Pasteurized Milk	Oval	Short chains and pairs
U 3	Standard Pasteurized Milk	Oval	Short chains and pairs
U 4	Standard Pasteurized Milk	Oval	Short chains and pairs

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General morphology and chain formation varied considerably among the various cultures of both groups. All cultures showed chain formation, but the proportion of the whole number of organisms in chains and the number of elements in the chains were points of variation. In some of the milk strains pairs or diplococci predominated, while in others practically all of the organisms were in chains of several elements. The human strains were not so variable in this respect. Most of them were in chains of varying lengths. Variations in the size and shape of the cells were also more marked in the milk strains. Some showed small and delicately spherical cells while others were larger and somewhat elongated.

The outstanding morphological differences between the organisms of the two groups may be summed up as follows:

MILK STRAINS:

Cells elongated-oval, varying greatly in size.

Organisms less regularly in long chains, and whether in chains or not, the arrangement of cells was quite uniformly in pairs.

HUMAN PATHOGENIC STRAINS:

Cells spherical, more regular in size and shape.

Chains usually long and few organisms not in chains. Some but not all showed a tendency to diplococcus form.

Some cultures in each group are indistinguishable morphologically from those of the other group but the majority

of each showed the differences outlined. These characters remained constant, when observed in the same medium, after months of cultivation.

The morphology alone is of little value in distinguishing bovine from human strains but when used with other characters, it will aid in the diagnosis of an unknown culture.

THE REACTION IN LITMUS MILK.

In the study of the reaction of streptococci in litmus milk, two distinct characters are involved, namely, the coagulation of the milk and the reduction of the litmus. Both have been found of some differential value by various observers. Observations which have been made with these characters are mentioned elsewhere. In addition it has been observed by several; Esten (49), Sherman (48), Evans (50), that the behavior of streptococci of the lacticus type in litmus milk is characteristic. This characteristic reaction is used in some laboratories of dairy bacteriology to identify members of this group. The typical *Streptococcus lacticus*, according to Evans, coagulates milk within twenty-four to forty-eight hours. This coagulation is accompanied by a reduction of the litmus, beginning at the bottom of the tube and progressing nearly to the surface, leaving a pure white column of milk with a pink layer on top.

All the cultures of human and milk strains of hemolytic streptococci were inoculated into litmus milk. The

tubes were filled to a uniform depth of about one and one-half inches and the litmus in all tubes was as nearly as possible of equal concentration. The inoculated tubes were incubated at 37° C. and observed at frequent intervals. Record was made of the reaction after twenty-four and forty-eight hours. The results are shown in Tables IV and V.

It will be seen that the majority of the milk strains produce acid and cause a reduction of the litmus in twenty-four hours. Some cause coagulation of the milk also, but in most cases this occurred between the twenty-four and the forty-eight hour readings. Reduction of the litmus in the bottom of the tube appeared, in most cases, before any color change took place and by the time sufficient acid was formed to cause a pink coloration, only a layer about one-eighth to one-fourth of an inch thick at the top remained colored, the rest being pure white. This shallow layer persisted for about three days and then deepened slowly until at the end of a week about half of the column of milk was pink. The hemolytic streptococci from milk, therefore, give the same litmus milk reaction as do the members of the S. lacticus group.

Not all of the milk strains give this typical reaction, however. A small number acidify the milk very slowly and cause no coagulation or reduction of litmus. Special mention should be made of the "W" strains. These are from a high grade of certified milk. This milk is produced under condi-

Table IV.

REPRODUCTION OF LARVAE WITHIN

IN 11 DAYS TIME.

Culture	REPRODUCTION AFTER	
	24 Hours	48 Hours
B 1	R A	R A C
B 2	R A	R A C
B 3	R A	R A C
B 4	R A	R A C
B 5	R A	R A C
F 6	R A	R A C
F 7	R A C	R A C
F 8	R A	R A C
F 9	R A	R A C
F 10	R A C	R A C
F 11	R A C	R A C
F 12	R A C	R A C
F 13	R A C	R A C
F 14	R A C	R A C
C 4	R A	R A C
C 7	R A	R A C
C 8	R A	R A C
C 9	R A	R A C
C 10	R A	R A C
W 1	No change	A
W 2	No change	A
W 3	No change	A
W 4	No change	A
W 5	No change	A
W 6	No change	A
W 8	No change	A
W 9	No change	A
D 6	R A C	R A C
D 7	R A C	R A C
D 8	R A C	R A C
D 9	R A C	R A C
D 10	R A C	R A C
D 11	R A C	R A C
D 12	R A C	R A C
U 1	R A C	R A C
U 2	R A C	R A C
U 3	R A C	R A C
U 4	R A C	R A C

R = Reduction of larvae.

A = Adult.

C = Cystulation.

Table V.

CHANGES OF BACTERIAL CULTURE

IN LITERS OF LIT.

Culture	EXPERIMENTAL PERIOD	
	24 hours	48 hours
24	No change	A
177	No change	A
179	No change	A
180	No change	No change
182	No change	No change
200	No change	A
233	No change	A
234	No change	A
240	No change	A
236	No change	No change
242	No change	A
247	A	A C
250	No change	No change
252	No change	No change
258	No change	A
259	No change	A
260	No change	A
261	No change	A
33	A	A
44	No change	No change
7	No change	No change
10	A	A C
190 (vir.)	R A C	R A C
DE	R A	R A C

R = Reduction of litmus.

A = Acid.

C = Coagulation.

tions which would, as nearly as possible by ordinary precautions, exclude organisms from such sources as the cow's flanks, the hand of the milker, the stable air, and unsterilized utensils. The organisms present would, therefore, be largely represented by those coming from the interior of the udder.

Sherman and Albus (48) considered the streptococci obtained from the udder of the cow to be of the pyogenes type and found them distinctly different, in a number of characters, from the lactic acid type. It is possible, therefore, that these milk strains which do not coagulate milk and reduce litmus are of the pyogenes type. The subsequent tests support this view.

The human pathogenic strains act slowly upon litmus milk. Little change is seen in twenty-four hours. Many of them show a weak acidity in forty-eight hours, while others cause little or no apparent change in the milk even after several days incubation. Reduction of litmus is brought about by but one strain, two strains cause coagulation. Two strains of S. viridans which were included for comparison cause both reduction and coagulation.

In a general way, hemolytic streptococci from milk produced under ordinary conditions bring about a rapid acidification, coagulation, and reduction in litmus milk, while human pathogenic strains only produce a slight acidity. The exceptions, however, are too numerous to use this test as absolute differentiation.

FERMENTATION OF CARBOHYDRATES.

The fermentation tests were made in a veal infusion broth which was prepared after the method described by Huntoon, that is, the broth was not filtered through cotton or filter paper but was allowed to clarify by sedimentation in tall jars. This medium gave abundant growth of all strains without the addition of carbohydrates. In preliminary tests the broth was inoculated with B. coli to rid it of muscle sugar, but by comparison with controls this proved to be unnecessary and was abandoned. In all tests, however, control tubes to which no carbohydrate was added were inoculated and tested with the rest.

The test substances were made up in ten per cent solutions and sterilized separately. One per cent of the test substance was added to the tubed and sterilized broth. The tubes were then incubated for twenty-four hours to insure sterility. Transfers were made to plain infusion broth from the stock cultures of the organisms to be tested. After twenty-four hours incubation, one-tenth cc. of this culture was added to each of the test broth tubes. All tubes were incubated seven days at 37° C., including the tube from which seeding was made, this tube being the control mentioned above. Two drops of Andrade's indicator were added to each tube at the end of the incubation period and the tube was allowed to stand for a few minutes before being read. Little difficulty was encountered with doubtful reactions, nearly all being

distinctly positive or negative. None of the controls showed any color with the indicator.

A tabular view of the results may be seen in Tables VI and VII. The only remarkable feature of these results is the uniformity with which saccharose is fermented by the human pathogenic strains and the negative results obtained with this sugar when tested with the milk strains. The exceptions are practically all in the "W" strains, which resemble the human strains in their fermentation reactions. It was noted in the preceding test that they also react in litmus milk as do the human pathogens.

These results are similar to those obtained by Sherman and Albus (48) who found that pyogenic streptococci fermented saccharose, while streptococci of the lacticus type did not. Their pyogenic cultures were isolated from the udders of cows and were probably like the "W" strains in these tables.

In the several classifications of streptococci based on fermentation this saccharose negative hemolytic streptococcus seems to have been overlooked. Andrews and Horder (7) have no saccharose negative groups, and Holman (27) does not use saccharose in his classification. In the latter classification, lactose and salicin positive, mannite negative, hemolytic streptococci such as are shown in the tables would fall into the S. pyogenes group. So also would the human

Table VI

RELATIONSHIP OF A PROTEIN TO THE QUALITY

U-Value	Dextrose	Inositol	Saccharose	Leucine	Glycine	L-Ala.	DL-Ala.	Control
U1	+	+	-	-	+	-	-	-
U2	+	+	-	-	+	-	-	-
U3	+	+	-	-	+	-	-	-
U4	+	+	-	-	+	-	-	-
U5	+	+	-	-	+	-	-	-
U6	+	+	-	-	+	-	-	-
U7	+	+	-	-	+	-	-	-
U8	+	+	-	-	+	-	-	-
U9	+	+	-	-	+	-	-	-
U10	+	+	-	-	+	-	-	-
U11	+	+	+	-	+	-	-	-
U12	+	+	+	-	+	-	-	-
U13	+	+	+	-	+	-	-	-
U14	+	+	+	-	+	-	-	-
U15	+	+	+	-	+	-	-	-
U16	+	+	+	-	+	-	-	-
U17	+	+	+	-	+	-	-	-
U18	+	+	+	-	+	-	-	-
U19	+	+	+	-	+	-	-	-
U20	+	+	+	-	+	-	-	-
U21	+	+	+	-	+	-	-	-
U22	+	+	+	-	+	-	-	-
U23	+	+	+	-	+	-	-	-
U24	+	+	+	-	+	-	-	-
U25	+	+	+	-	+	-	-	-
U26	+	+	+	-	+	-	-	-
U27	+	+	+	-	+	-	-	-
U28	+	+	+	-	+	-	-	-
U29	+	+	+	-	+	-	-	-
U30	+	+	+	-	+	-	-	-
U31	+	+	+	-	+	-	-	-
U32	+	+	+	-	+	-	-	-

Table VII

FRANKLIN D. JENNINGS

pathogenic strains, and these two groups would not be differentiated.

It appears from the literature that the majority of milk streptococci ferment saccharose. Streptococcus lacticus is so described in text books. Savage (3) stated that most milk streptococci ferment saccharose. Houston (6) found this true of ninety per cent of milk streptococci, while Broadhurst (13) found only sixty-six per cent of milk strains saccharose fermenters.

Sherman and Albus (48), on the other hand, believe that typical Streptococcus lacticus does not ferment saccharose and their experiments seem to show that the fermentation of this sugar distinguishes S. pyogenes of cows' udders from S. lacticus.

From the data presented in Tables V and VI, it appears that saccharose may be useful in differentiating non-pathogenic hemolytic streptococci of milk from those of human origin.

HEMOLYSIS.

As stated before, all of the strains used in this study were isolated by picking typical hemolytic colonies from blood agar plates. In so doing only those colonies were selected which gave the Beta type of hemolysis (Smith and Brown). In the appearance on blood agar plates, therefore, all of the milk strains were practically identical and they proved to be identical in appearance with the human pathogenic

strains when grown on blood agar plates prepared in the same manner. The two groups were therefore indistinguishable by blood agar plate hemolysis.

All cultures were then further tested for the production of a soluble hemolysin in broth as recommended by the Medical Department of the United States Army in a leaflet entitled, "The Isolation and Identification of Streptococcus Hemolyticus." The cultures were grown in infusion broth for eighteen to twenty-four hours. One-half cc. of broth culture was then added to one-half cc. of five per cent saline suspension of washed rabbit red blood cells and incubated for two hours. Positive controls of known hemolytic cultures and negative controls with sterile broth were included in each test. Only those cultures which gave complete hemolysis in two hours were retained for study. All of the colonies which gave a wide, absolutely clear zone with well-defined limits responded to this test. Occasionally colonies were picked which gave wide, clear zones of hemolysis, but which, on close inspection showed some red color remaining in the zone. These cultures always gave negative results in broth. Others again had wide, clear zones but the outer border was not sharply defined. These also gave negative results in broth.

After some study one may predict with considerable accuracy the reaction in broth by the appearance of the zone of hemolysis on a standard blood agar plate. The production

of hemolysin in broth culture, therefore, seems to correlate with a definite appearance on blood agar plates, namely, the Beta type of hemolysis. This distinguishes a group of hemolytic streptococci with definite hemolytic properties. Clear-cut results, that is, distinctly positive or negative, are obtained with this test.

It seems preferable to use this test to confirm results obtained with the blood agar plate. Variations in technique and in the personal judgment of different investigators render the reports of types of hemolysis on blood agar plates too uncertain. If a culture is reported as positive or negative in the broth test, its hemolytic properties are more clearly defined. Swabs were taken from ten normal throats of the members of a class in bacteriology. All showed hemolytic colonies in varying numbers. The zones of hemolysis were wide, with well-defined borders. They were hardly distinguishable from the true Beta type. None of the ten, however, gave positive results in broth cultures. This suggests that perhaps the widely varying results reported in regard to the occurrence of hemolytic streptococcus in normal throats may be due to variations in what are considered to be true hemolytic streptococci.

It is evident that neither of the hemolysin tests mentioned suffices to distinguish Beta hemolytic streptococci from milk and from human lesions. Brown, in a recent paper, noted that cultures of milk strains of the Beta type require a longer period of incubation with the red cells suspension

to produce hemolysis. This fact had often been observed in the present work and an experiment was planned to compare the two groups of cultures in this respect, also to compare their hemolytic titer. Tables VIII and IX show the results of the test. The cultures were diluted with saline, incubated at the same time in a 37° C. water bath and the results read at the end of thirty and ninety minutes. It will be seen that the milk strains have a very low titer after thirty minutes, while the human strains show an almost complete hemolysis. The one "W" strain included resembles the human strains as in previous tests. After ninety minutes there is no striking difference in the hemolytic titer of the two groups. These results agree with those of Brown (3), and it is evident that the rate of lysis of blood cells is more rapid with human strains.

Another observation in connection with tube hemolysis of the milk strains should be noted, namely, the production of brownish discoloration of the cells before they are laked. This occurred with all of the milk strains although it was more pronounced in some than in others. The color remained and intensified after the lysis of the cells. Occasionally some of the human strains showed a slight browning of the hemoglobin. Spectroscopic examination of the brown colored tubes showed the characteristic absorption bands of methemoglobin in the red, yellow, and green.



Table VIII.

BUREAU OF HEALTH AND HUMAN SERVICES

Neurites after third division.

Latitude	Longitude					Latitude	Longitude				
	1/2	1/4	1/8	1/16	1/32		1/2	1/4	1/8	1/16	1/32
U 1	++	+				174	+++	++			
U 2	++	+				177	+++	+++	+++	++	
U 3	+					179	+++	+++	+++	+++	
W 1	+++	+++	+++			180	+++	+++	+++	+++	+++
F 7	++	+				182	+++	+++	+++	+++	+++
F 8	+					183	+++	+++	+++	+++	
F 9	+	+				184	+++	+++	+++	++	
F 10	++					185	+++	++	+++	++	+
C 0	++					186	+++	+++	+++	++	+
Q 10	++					187	+++	+++	+++	++	
D 6	+					188	+++	+++	+++	+++	+++
D 7	++	+				189	+++	+++	+++	+++	+++
D 8	+++	+++	+			190	+++	+++	+++	+++	+++
D 12	++	+				191	+++	+++	+++	+++	+++
W 2	+++	++				192	+++	+++	+++	+++	+++
W 3	++	+				193	+++	+++	+++	+++	+++
B 3	+++	++	+			194	+++	+++	+++	+++	+++
B 4	++					195	+++	+++	+++	+++	+++
B 5	++	+				196	++	++	+		
B 1	+					197	++	++	+		
D 10	+					198	++	++	+		
D 9	+					199	++	++	+		
W 4	+++	++				200	++	++	+		
W 5	+++	+++	+++	+++	+++	201	++	++	+		
D 11	+++	++	+			202	++	++	+		
F 12	++	+				203	++	++	+		
F 11	+	+				204	++	++	+		

Table IX.

DATA ON BACTERIAL GROWTH AND TOXIN PRODUCTION

(Incubation after ninety minutes.)

Inoculum	DURATION					Human Culture	TOXIN				
	1/2	1/4	1/2	1/16	1/32		1/2	1/4	1/2	1/16	1/32
1	+++	+++	++	++	+	24	+++	+++	+++	++	+
2	+++	+++	+++	+++	++	170	+++	+++	+++	+++	++
3	+++	+++	+++	+++	++	175	+++	+++	+++	+++	++
7	+++	+++	+++	+++	+++	180	+++	+++	+++	+++	++
1	+++	+++	+++	+++	++	182	+++	+++	+++	+++	+++
8	+++	+++	+++	++	+	185	+++	+++	+++	+++	++
9	+++	+++	+++	++	+	188	+++	+++	+++	+++	++
0	+++	+++	++	++	+	190	+++	+++	+++	+++	++
8	+++	+++	+++	++	+	192	+++	+++	+++	+++	++
0	+++	+++	+++	++	+	195	+++	+++	+++	+++	++
6	+++	+++	+++	+++	+++	200	+++	+++	+++	+++	+++
7	+++	+++	+++	+++	+++	205	+++	+++	+++	+++	+++
8	++	+++	+++	+++	+++	210	+++	+++	+++	+++	+++
2	+++	+++	+++	+++	+++	215	+++	+++	+++	+++	+++
2	+++	+++	++	+	+	220	+++	+++	+++	+++	+++
3	++	++	+	+	+	225	+++	+++	+++	+++	+++
3	+++	+++	+++	+++	+++	230	+++	+++	+++	+++	+++
4	+++	+++	+++	+++	+++	235	+++	+++	+++	+++	+++
5	+++	+++	+++	+++	+++	240	+++	+++	+++	+++	+++
1	+++	+++	+++	+++	+++	245	+++	+++	+++	+++	+++
0	+++	+++	+++	+++	+++	250	+++	+++	+++	+++	+++
9	+++	+++	+++	+++	+++	255	+++	+++	+++	+++	+++
8	+++	+++	+++	++	+	260	+++	+++	+++	+++	+++
6	+++	+++	+++	+++	+++	265	+++	+++	+++	+++	+++
5	+++	+++	+++	+++	+++	270	+++	+++	+++	+++	+++
1	+++	+++	+++	+++	+++	275	+++	+++	+++	+++	+++
1	+++	+++	+++	+++	+++	280	+++	+++	+++	+++	+++
1	+++	+++	+++	+++	+++	285	+++	+++	+++	+++	+++
1	+++	+++	+++	+++	+++	290	+++	+++	+++	+++	+++
1	+++	+++	+++	+++	+++	295	+++	+++	+++	+++	+++
1	+++	+++	+++	+++	+++	300	+++	+++	+++	+++	+++

PATHOGENICITY FOR MICE.

Streptococci vary so greatly in their pathogenicity for animals that the value of inoculation experiments may easily be over-estimated. It is very difficult to get constant results even with cultures of known pathogenicity. Thus mouse protection tests of antistreptococcic serums have not proven of great significance in judging the value of the serum, because of the inconstancy of control inoculations. The inoculation of animals with a few strains of a group of streptococci might easily lead to erroneous conclusions, but when a large number of organisms of similar properties give constant results some conclusion may be warranted.

The work attempted here was to show whether any pathogenicity was demonstrable in the hemolytic streptococci obtained from milk. Davis (2) reports some pathogenicity for rabbits in a few cultures, but his results are generally negative.

Most of the cultures isolated were inoculated into mice. This was done as soon as possible after isolation. As soon as their purity was assured the organisms were given three daily transfers in serum broth, then one-half cc. of the third broth culture was inoculated into the peritoneal cavity of a mouse. The human pathogens were treated in the same way. All mice were observed daily for seven days and doubtful results were repeated. In no case were any of the

Table X.

Culture	Time after inoculation				
	14 hours	24 hours	36 hours	7 days	100% (100%)
U 1	Normal	Normal	Normal	Normal	
U 2	Normal	Normal	Normal	Normal	
U 3	Normal	Normal	Normal	Normal	
U 4	Normal	Normal	Normal	Normal	
U 7	Normal	Normal	Normal	Normal	
U 8	Normal	Normal	Normal	Normal	
U 9	Normal	Normal	Normal	Normal	
U 10	Normal	Normal	Normal	Normal	
W 1	Sick	Sick	Sick	Normal	
W 2	Sick	Sick	Sick	Normal	
W 3	Sick	Sick	Sick	Normal	
W 4	Sick	Sick	Normal	Normal	
W 5	Sick	Sick	Sick	Normal	
W 6	Dead				+
W 8	Normal	Normal	Normal	Normal	
W 9	Sick	Sick	Sick	Normal	
C 7	Normal	Normal	Normal	Normal	
C 8	Normal	Normal	Normal	Normal	
C 9	Normal	Normal	Normal	Normal	
C 10	Normal	Normal	Normal	Normal	

Table XI.

RESULTS OF VACCINATION TESTS
PERFORMED IN CHINA.

Culture	Time after inoculation					Remarks
	10 hours	24 hours	36 hours	7 days	10 days	
24	Normal	Normal	Normal	Normal		
177	Normal	Normal	Normal	Normal		
179	Normal	Normal	Normal	Normal		
180	Normal	Normal	Normal	Normal		
182	Dead					+
200	Dead					+
233	Dead					+
234	Dead					+
238	Sick	Dead				+
240	Dead					+
242	Sick	Dead				+
260	Dead					+
250	Dead					+
252	Dead					+
258	Sick	Dead				+
259	Dead					+
261	Dead					+
247	Sick	Sick	Dead			+
186(vir.)	Normal	Normal	Normal	Normal		
30	Dead					+
7	Sick	Sick	Dead			+
18	Normal	Normal	Normal	Normal		
44	Dead					+
45	Dead					+
51(vir.)	Sick	Dead				+
Repeated tests						
24	Sick	Dead				+
177	Sick	Dead				+
179	Sick	Sick	Dead			+
180	Sick	Normal	Normal	Normal		
232	Dead					+
234	Dead					+
240	Sick	Dead				+
242	Sick	Sick	Dead			+
186(vir.)	Sick	Normal	Normal	Normal		

typical milk strains pathogenic for mice. One of the "M" strains killed mice and all of them seemed to cause rather severe symptoms. The back would be arched, the hair roughed, and respirations rapid and labored. This seems to confirm the suspicion that these strains are of the pyogenic type and resemble more closely the human pathogenic than the milk strains. All of the former group showed definite pathogenicity for mice. Postmortem examination was made of all mice that died and the heart blood and peritoneal fluid streaked on blood agar plates and inoculated into broth. The organisms thus isolated were always identical morphologically and in their hemolytic properties with those injected. It would seem that the milk strains were not pathogenic for mice. A number of them were injected intravenously into rabbits with entirely negative results.

Heinemann (36) reported that S. lacticus could be made virulent for rabbits by successive subcutaneous inoculations. The organism was re-isolated from the site of inoculation and re-injected into another rabbit and so on. The lesions were said to become more severe until a culture was obtained, after several transfers, which killed rabbits. Although the culture was examined and identified after each isolation, the danger of involving a streptococcus from the body of the animal is not excluded.

Typical milk strains of hemolytic streptococci were

injected successively into six mice. The mouse was etherized in each case, two days after the inoculation and the streptococci isolated from the peritoneal fluid, and occasionally from the heart blood. These were re-injected into the next mouse and so on. One-half cc. of broth culture was injected into each mouse. The organisms showed no signs of increasing in virulence, the mice in all cases appearing normal. It does not seem possible, therefore, to render a strain of the hemolytic streptococci from milk virulent by passage through mice.

THERMAL DEATH RATES.

The statement is made by Ayers, Johnson, and Davis (46) that pathogenic streptococci are killed at 60° C. when held for thirty minutes in milk and that if any survive this heating, they are so attenuated as not to be dangerous. Some of their cultures which survived this temperature, although coming from pathological sources, were not considered pathogenic. The question is thus raised as to whether or not there is a decided difference in the thermal death rate of the pathogenic and non-pathogenic streptococci. If true, this would settle any doubts in regard to efficiency of pasteurization with respect to streptococci and, furthermore, might offer a convenient means of differentiation between the pathogenic and non-pathogenic streptococci in milk.

Some recent publications have emphasized the fallacy

of speaking of thermal death points when all of the factors governing the complete sterilization of the culture are not controlled. It has been repeatedly demonstrated that the time required to kill all the organisms at a given temperature is dependent on the initial number present. According to Phelps (51), "the rate of dying whether under the influence of heat, cold, or chemical poison, is unfailingly found to follow the logarithmic curve of the velocity law, if the temperature be constant." This curve never reaches zero value although approaching it indefinitely. He further states that thermal death points "are merely accidental, depending in the first place wholly on the technique adopted and again, upon the chance of the last few survivors living or dying, when for a considerable length of time these chances are about equal."

Any method, therefore, which is designed to give a refined comparison between the effect of heat upon different cultures must take into consideration the initial number of organisms and compare, not the temperature at which all organisms are killed in a given time, but the rate of death at a given constant temperature. In other words, the velocity coefficients of the rates of death of the organisms should be compared. Bruett (52) used these velocity coefficients, or K values, determined for spore bearing organisms, before and after "cold shock" in order to determine the utility of "blanching" in food canning. This method made possible a

refined comparison of the rates of death of cultures of the same species when differently treated. Differences in heat resistance between the various groups of streptococci ought, therefore, to be demonstrable by this method. It is conceded that the determination of the thermal death point will show differences in heat resistance among streptococci, but in this determination only complete sterilization is considered. The accidental survival of a single or a few organisms is not distinguished from the survival of all or a large proportion of the initial number and thus distinct differences in behavior of two organisms at a given temperature may be obscured.

It has been sufficiently demonstrated that bacteria when subjected to fixed unfavorable conditions such as a constant high temperature, will die off in a manner corresponding to the law of monomolecular reactions. This law is expressed by the equation:

$$K = \frac{1}{t} \log \frac{B}{b}$$

in which K is a constant, the velocity coefficient of the rate of death,
t is the time between determinations of number of organisms,
B is the initial number of bacteria in any time interval,
b is the number of bacteria at the end of time t.

In this formula the K varies only with the temperature, is independent of the initial number of organisms, and does not involve complete sterilization. The point of complete sterilization is indefinite, therefore the K is more accurately

determined when b is not near the point of complete sterilization. The best results are obtained, according to Phelps (51), when there is about a fifty per cent reduction in the number of organisms.

It was thought best to determine the velocity coefficients of the rate of death of the hemolytic streptococci in milk, since the application of the results to pasteurization, as well as to the differentiation of the organisms, was sought. Each strain was grown in milk for three days, transfer being made each day. The third twenty-four hour culture was used for the test. The heating was done in a De Khotinsky electric bath in which the temperature was controlled very accurately. A tube containing ten cc. of sterile milk was placed in the bath and held for five minutes. This was found sufficient to raise the milk to the temperature of the bath. The organisms were then pipetted into the tube from the twenty-four hour culture. It was found that with the milk cultures one-tenth of a cubic centimeter of the twenty-four hour culture added to the heated milk tube gave a suspension containing between one and ten million bacteria per cc. With the pathogenic strains one cc. of the twenty-four hour culture gave about the same number. The latter, therefore, did not seem to develop so abundantly in milk. The difference in counts, however, may have been due in part to the presence of longer chains in the pathogenic strains, with the result that more individuals formed the seed of a single colony.

After the organisms were added the tube was shaken

and one cc. removed for the initial count. The milk strains were enumerated in dextrose infusion agar, but it was found necessary to use whole blood agar to count the pathogenic strains. The colonies were so small on the infusion medium that there was danger of overlooking some of them, while on blood agar the zones of hemolysis around each colony made counting easy. Four plates were made for each count. Two dilutions were plated, each one in duplicate. Counts which did not check satisfactorily both in the two dilutions and in the duplicates of each were discarded and the determination repeated.

The tubes while being heated were kept immersed to a point at least two inches above the level of the milk. Before each count the milk was mixed by rolling the tube vigorously between the palms, as far as possible without removing the tube from the water bath. Care was taken to avoid splashing the milk high up on the walls of the tube or on the cotton plug, as in this way some of the organisms might escape being heated to the required temperature.

The test of the applicability of the law of monomolecular reactions to this work is the constancy of the K values at a given temperature. A number of preliminary determinations were made at seven and one-half minute intervals with a group of cultures, in order to test the constancy of the K values. The results are best shown by graphs. If the logarithms of the

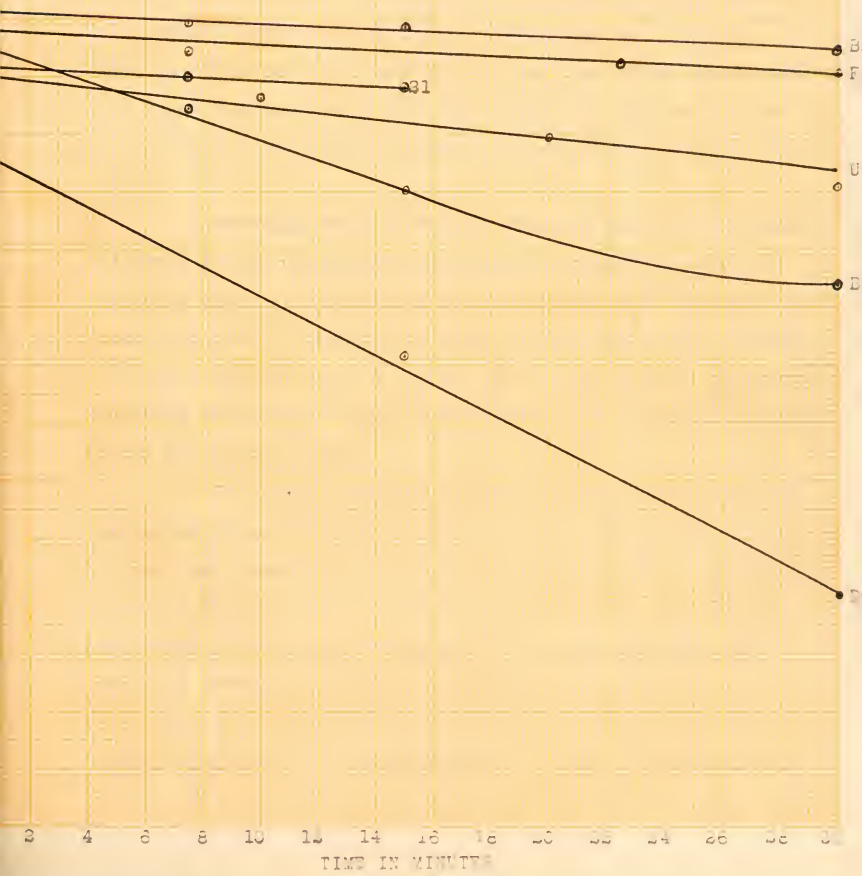
numbers of organisms determined at various intervals, in a culture held at constant temperature, are plotted against time, a line may be drawn, the slope of which is dependent upon K . If the K values are constant for determinations at all intervals of time, the organisms are dying at a constant rate and the plot should therefore be a straight line. By plotting the logarithms of the numbers we may test the constancy of K without determining it. Graphs plotted from some of the data are shown in Figure I. It will be seen that the K values do not vary greatly, as most of the plots approximate a straight line. Subsequent determinations with other cultures did not always yield such uniform results, but the variations from straight lines are quite similar in all and can be explained on the same basis.

It frequently occurs in these cultures that the velocity coefficient of the rate of death during the first interval of time is not as great as in the second and third, also that as the number of organisms gets very small there is considerable variation in the coefficient. The most constant results are obtained when the decrease in the number of organisms does not go much beyond fifty per cent. This may be due to the greater percentage of experimental error in counting small numbers of organisms, or perhaps the law is no longer strictly operative when small numbers are reached.

The slow rate in the first interval of time has two

FIGURE 1.

GRAPHS SHOWING THERMAL DEATH RATE OF HEMOLYTIC STREPTOCOCCI
IN MILK AT 60° C.



possible explanations. First, a slight error is introduced by the addition of the suspension of the organisms to the heated milk, causing a slight reduction in temperature. The first counts were made as soon after the introduction of the organisms as the heated milk suspension could be properly mixed. In this way, the sample may have been taken occasionally before the proper temperature was regained. Second, shaking after the first heating interval may cause the breaking up of chains, making a larger number of colonies for the actual number of bacteria present than appeared in the first count.

Although the K's are not constant for all intervals of time, it was considered that the best comparison of the rates of death of two groups of cultures could be obtained by comparing the K's computed for the longest practicable length of time. Most of the K's for the milk strains were, therefore, computed for thirty minutes and those of the pathogenic strains for a ten minute period.

It becomes apparent from an examination of the data in Tables XII and XIII that there is a very marked difference between the behavior of the human and milk hemolytic streptococci in milk at 60° C. The human strains die very rapidly and reach a very small number per cc. in ten minutes, while the milk strains die very slowly at this temperature, some in fact appear not to decrease to any appreciable extent in thirty minutes. It is questionable, because of the small num-

RATES OF DEATH OF MILK CULTURES IN MILK AT 60° C.

Culture	Initial number of bacteria per cc.	NUMBER OF BACTERIA PER CC. AFTER			K
		10 minutes	20 minutes	30 minutes	
		10 minutes	20 minutes	30 minutes	
V 1	9 700 000	-	-	1 000 000	.0029
V 2	3 300 000	2 100 000	950 000	342 000	.0027
V 7	2 450 000	7 250 000	6 600 000	5 170 000	.0071
V 3	9 300 000	9 650 000	9 900 000	8 300 000	.0014
V 4	11 200 000	11 000 000	-	10 100 000	.0014
V 6	5 300 000	4 500 000	3 600 000	3 300 000	.0056
V 9	4 400 000	4 400 000	3 500 000	3 300 000	.0024
C 7	4 500 000	4 500 000	4 000 000	3 600 000	.0014
C 6	7 000 000	1 400 000	90 000	50 000	.0014
C 9	7 100 000	2 900 000	1 600 000	1 300 000	.0020
C 10	7 000 000	2 900 000	1 390 000	-	.0021
F 11	3 500 000	1 000 000	1 000 000	25 000	.0020
F 12	4 300 000	4 500 000	3 400 000	2 700 000	.0072
F 8	3 900 000	27	-	-	.010
F 6	2 500 000	240	-	-	.0017
F 9	35 000 000	26	-	-	.0020
F 2	4 100 000	0	-	-	-
F 3	500 000	110	-	-	.0020
F 4	3 400 000	107	-	-	.0020

RANGE OF GRAIN OF FERTILIZING CULTURES IN MIN AT 60 C.

Culture	Initial number of bacteria per cc.	Initial number of bacteria per cc. after 10 minutes	F
177	2 000 000	140	.0021
250	5 200 000	60	.0037
256	4 200 000	191	.040
182	61 000	47	.0007
230	1 400 000	11 000	.008
259	5 000 000	540	.0106
180	450 000	0	-
231	3 300 000	210	.0039
242	11 500 000	245	.0037
252	300 000	2 500	.002
253	2 000 000	90	.015
260	200 000	245	.0039
179	700 000	55	.0152
250	4 200 000	101	.0067
247	900 000	16	.0003
252	255 000	2 500	.0003
24	970 000	14	.0001
250	5 200 000	24	.0104

ber of organisms left after the time interval, whether or not the K values for the pathogenic streptococci represent the rapidity of the rate of decrease. The curve of decline of numbers probably reaches a point, before the ten minutes have elapsed, where death is no longer in strict accordance with the law of monomolecular reactions. In other words, it reaches that small number in which chance plays an important part in survival. The determination of the K's, however, obviously gives a better picture of the behavior of the organisms than a determination of the point of complete sterilization, the undeterminable point at which all of these few remaining organisms have died.

The rates of death of the pathogenic strains are fairly uniform as compared with the milk strains, although none of the typical strains of either have a death rate which approaches that of the other group. The "W" strains, as in previous tests, show great similarity to the pathogenic strains. In all of the tests they have been identical with the pathogenic group and there seems to be little reason to believe that they are not of this group. None of these organisms were obtained from pasteurized milk but from milk produced under the best of sanitary precautions. It would be difficult to prove whether or not these strains could be pathogenic to man, but their similarity to pathogenic strains and the fact that they were found only in unpasteurized milk argues very strongly for pasteurization of all milk regardless

of the care used in producing it.

The great difference in the thermal death rate of pathogenic human and that of non-pathogenic milk streptococci suggests that this character might be added to the list of characters which serve to differentiate them. It is one which can easily be determined with simple apparatus. It is also a character which perhaps reflects the habitual environment of the organism, that is, a purely saprophytic organism would be more likely to withstand wide ranges of temperature than one which lives and grows largely at the temperature of the human or other animal body.

The question as to whether or not pathogenic streptococci might survive pasteurization arose in connection with these experiments. The usual pasteurizing temperature is 145° F. or 62.8° C., although 60° C. is occasionally used. Ayers, Johnson, and Davis (46) concluded that pathogenic streptococci will not survive this temperature for thirty minutes.

All of the pathogenic strains used in the above tests were held at 60° C. in milk for thirty minutes. All strains were tested several times. The tubes were cooled after heating and incubated for forty-eight hours at 37° C. Occasionally some of them survived, grew in the milk, and were carefully identified. In these tests the initial number of organisms was the same as that in the determination of the death rate, that is, one cc. of a twenty-four milk culture was added to a tube containing about ten cc. of milk at 60° C. Some of

the cultures which grew after the one heat test failed to grow after a second test. Others, namely, 33, 44, DB, and L1, (*S. viridans*), grew quite consistently after heating for thirty minutes at 60° C. If the initial number of organisms were reduced to one-tenth cc. of an twenty-four hour milk culture, none of the cultures survived the test.

The question arose as to whether the organisms lost their virulence by the heating. To test this, hemolytic culture No. 33 and viridans culture L1 were inoculated into mice. Both mice died in twenty-four hours. The organisms were isolated from the heart blood on blood agar plates and identified. A single colony of each was transferred to a milk tube and incubated for twenty-four hours. One cc. was transferred from each of the tubes to sterile milk tubes at 60° C. and held for thirty minutes. The tubes were then cooled and incubated for twenty-four hours. One-half cc. of each was injected intraperitoneally into a mouse and a third mouse was inoculated with one-half cc. of sterile milk. Both of the mice inoculated with the heated streptococci cultures died within forty-eight hours, while the control survived. The organisms were again isolated from the heart blood and identified. This simple experiment seems to show that the virulence of the streptococci which survive pasteurization is not lost, or at least they are able to produce virulent cells in the same milk in which they were heated. The experiment was repeated with

the same results.

It must be remembered that in these experiments the number of organisms present was very large. There were probably from one to five million bacteria per cc. present at the beginning. One would scarcely expect to find milk so highly contaminated with pathogenic streptococci. However, the margin of safety in pasteurization would be much more adequate if higher pasteurizing temperatures were used.

It was thought that repeated heating of a culture might lead to the selection of a heat resistant strain of the organisms. Accordingly, two hemolytic cultures, No. 33 and DB, and viridans culture L1 were pasteurized at 60° C. for thirty minutes, every second day for a time. It was intended to heat them ten times and then determine their death rates and compare them with those of the original cultures to see if the heat resistance of the organisms had increased. None of the three cultures, however, survived for ten heatings. The viridans strain was killed by the sixth heating, the hemolytic strain No. 33 by the eighth, and DB by the ninth. It seems clear that the cultures did not gain in heat resistance by the repeated heatings. The apparent decrease in resistance after a number of heatings could not be explained.

CONCLUSIONS.

The number of cultures used in this work was not great, especially the group of human pathogenic cultures, yet they were carefully selected and are believed to be fairly representative. Several conclusions may, therefore, be warranted from the study of the data presented.

1. Hemolytic streptococci which are identical in hemolytic power with the human pathogenic type but which differ from them in many respects, occur quite generally in raw and pasteurized milk. A large number of these strains behaved alike in a series of tests and they therefore seem to form a homogeneous group or species.

2. There are certain general differences in morphology between milk and human hemolytic streptococci. These are not absolute and by themselves are of little value for purposes of differentiation.

3. The reaction of milk hemolytic streptococci in litmus milk is characteristic and is of importance in differentiating them from pathogenic strains.

4. The pathogenic hemolytic streptococci have a greater range of fermenting power than do the milk strains. The most constant difference between the two groups is the failure of the latter to ferment saccharose.

5. The identification of hemolytic streptococci should always be confirmed by testing for hemolysin in broth cultures. The true Beta type produces hemolysin in



broth, while others which resemble the Beta type quite closely do not.

6. There is no constant difference in the hemolytic titer of milk and pathogenic strains, but the time required for hemolysis to take place is distinctly greater with the milk strains. This characteristic could be used to aid in differentiation.

7. The milk hemolytic streptococci are not pathogenic for mice, and virulence cannot be demonstrated even by successive passages.

8. The difference in the thermal death rates of milk and pathogenic hemolytic streptococci serves to differentiate them. The former die at a slow rate in milk at 60° C., while the latter die rapidly.

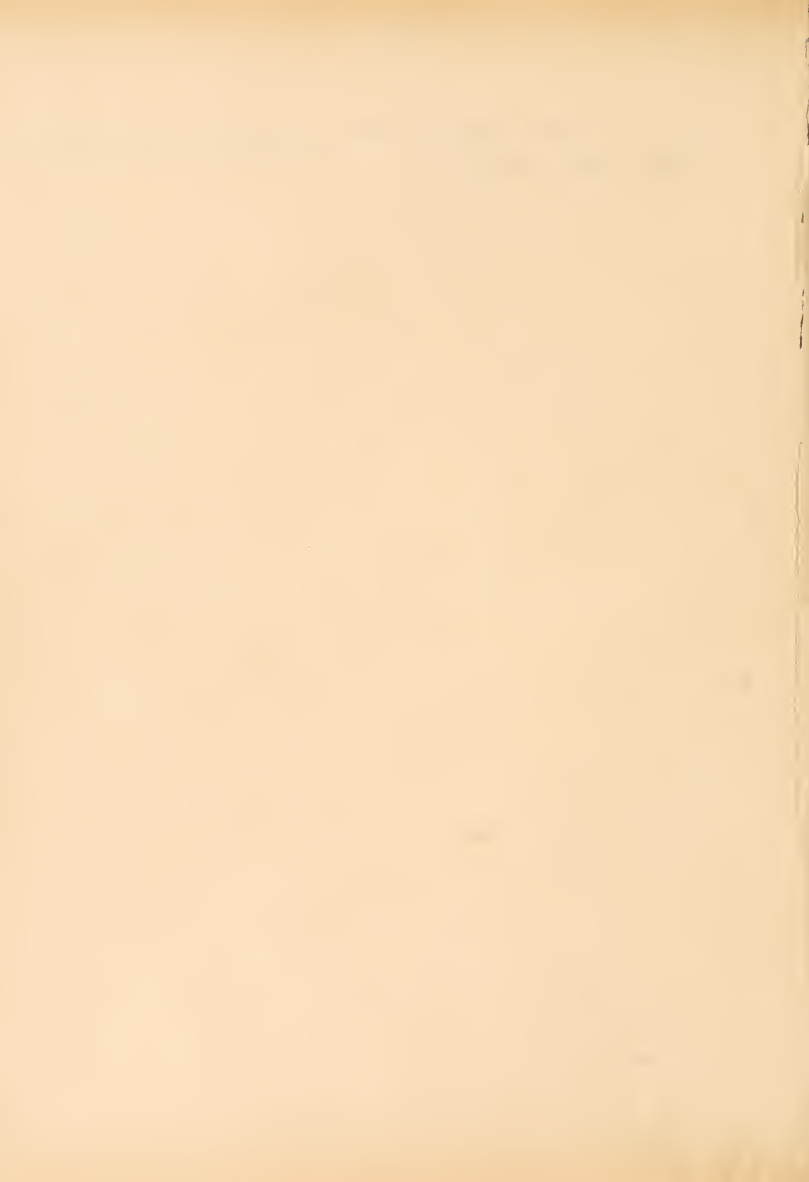
9. With a very large number present it is possible for pathogenic streptococci to survive pasteurization at 60° C. for thirty minutes. The number of survivors is undoubtedly very small, but they are able to produce a culture virulent for mice. Higher pasteurizing temperatures would give a better margin of safety.

10. Successive heating of pathogenic streptococci does not result in the production of a heat resistant strain.

11. Hemolytic streptococci resembling the pathogenic type in every respect were isolated from certified milk. They



are of low heat resistance and could readily be destroyed by proper pasteurization.



BIBLIOGRAPHY.

1. Smith, T. and Brown, J. H., J. Med. Research, 1915, 31, 445.
2. Davis, D. J., J. Infect. Dis., 1918, 23, 559.
3. Brown, J. H., J. Exper. Med., 31, 1, 35.
4. v. Lingelsheim, W., Beitr. z. Exp. Ther. Hft., 1, 1899.
5. Gordon, M. H., Rept. of Med. Off. to Loc. Govt. Bd.,
1903-1904, 393.
6. Houston, A. C., Rept. of Med. Off. to Loc. Govt. Bd.,
1904-1905, 326.
7. Andrews, T. W. and Horder, T. J., Lancet, 1906, 2, 708,
775, 852.
8. Savage, W. G., Rept. of Med. Off. to Loc. Govt. Bd.,
1906-1907, 206 and 1907-1908, 359.
9. Winslow, C. E. A. and Palmer, G. F., J. Infect. Dis.,
1910, 7, 1.
10. Winslow, C. E. A., J. Infect. Dis., 1912, 10, 285.
11. Stowell, E. C., Hilliard, C. M. and Schlessinger, M. J.,
J. Infect. Dis., 1913, 12, 144.
12. Fuller, C. A. and Armstrong, V. A., J. Infect. Dis.,
1913, 13, 442.
13. Broadhurst, Jean, J. Infect. Dis., 1913, 13, 404.
14. Thro, W. C., J. Infect. Dis., 1915, 17, 227.
15. Holman, W. L., J. Infect. Dis., 1914, 15, 293.
16. Kligler, I. J., J. Infect. Dis., 1915, 16, 327.
17. Hopkins, J. G. and Lang, Arvilla, J. Infect. Dis., 1914,
15, 63.
18. Shottmuller, H., Munch. Med. Woch., 1903, 1, 849.



19. Zangemeister, W., Deutsch. Med. Woch., 1919, 35, 427 and 477.
20. Hoessli, Hans, Centralbl. f. Bakteriol. Ite. Abt., Orig., 1910, 55, 135.
21. Rolly, Frank, Centralbl. f. Bakteriol. Ite. Abt., Orig., 1912, 56, 86.
23. Saito, Y., Arch. f. Hyg. u. Infektionskrankh., 1912, 75, 86.
23. Jaffe, R., Arch. f. Hyg. u. Infektionskrankh., 1912, 76, 97.
24. Kuhn, F., Centralbl. f. Bakteriol. Ite. Abt., Orig., 1912, 63, 97.
25. Ruediger, G. F., Science, 1912, 35, 223.
26. M'Leod, J. W., J. Pathol. and Bacteriol., 1914, 14, 392.
27. Holman, W. L., J. Med. Research., 1916, 34, 177.
28. Smillie, W. G., J. Infect. Dis., 1917, 20, 45.
29. Becker, W. C., J. Infect. Dis., 1916, 19, 754.
30. Brown, J. H., Monograph 9, Rockefeller Inst. for Med. Research.
31. Frankel, E., Munch. Med. Woch., 1915, 52, 548, 1868.
32. Ruediger, G. F., J. Infect. Dis., 1906, 3, 755.
33. Horder, T. J., Quart. J. Med., 1919, 2, 289.
34. Sachs, E., Zeit. f. Hyg. u. Infektionskrankh., 1919, 63, 463.
35. Bahr, Joseph, Arch. f. Hyg., 1910, 72, 91.
36. Heinsman, P. G., J. Infect. Dis., 1915, 16, 221.
37. Davis, D. J. and Rosenow, E. C., J. Am. Med. Ass., 1912, 58.
38. Stokes, W. R. and Hachtel, F. W., Bull. Hyg. Lab., U. S. P. H., 1912, 27, 47, 1923.
39. North, C. E., White, B. and Avery, O. T., J. Infect. Dis., 1914, 14, 124.

40. Capps, J. A. and Davis, D. J., J. Infect. Dis., 1914, 15, 130
41. Davis, D. J., J. Infect. Dis., 1913, 12, 386.
42. Krumweide, C. Jr. and Valentine, E., J. Med. Research,
1915, 33, 231.
43. Kinsella, R. and Swift, J. Exper. Med., 1918, 28, 169.
44. Howell, Katherine, J. Infect. Dis., 1918, 22, 230.
45. Havens, L. B., J. Infect. Dis., 1919, 25, 315.
46. Ayers, S. H., Johnson, W. T. and Davis, D. J., J. Infect.
Dis., 1918, 23, 291.
47. Avery, O. T. and Cullen, G., J. Exper. Med., 1919, 29, No. 3.
48. Sherman, J. and Albus, H., J. Bact., 1918, 3, 153.
49. Eaten, W. M., Conn. Storrs. Agr. Exp. Sta., Bul. 59.
50. Evans, Alice C., J. Agr. Research, 13, 235.
51. Phelps, E. D., J. Infect. Dis., 1911, 8, 27.
52. Bruett, Eva M., J. of Indus. and Eng. Chem., 1919, 2, 37.

BIOGRAPHY.

Raymond C. Salter was born at Chilton, Wisconsin, April 15, 1890. He attended the public schools of that city and was graduated from the High School in June, 1910. He entered the University of Wisconsin in September, 1911 and received the degree of Bachelor of Science in June, 1915, having majored in bacteriology.

During the years 1916 and 1917 he held the position of Instructor in Natural Science in the Iowa State Teachers College, Cedar Falls, Iowa. He was granted a year's leave of absence from that position in order to accept a fellowship in bacteriology at the Iowa State College, Ames, Iowa. The degree of Master of Science was conferred upon him in June, 1918.

Upon completion of his work at Ames he accepted an appointment as Sanitary Bacteriologist in the United States Public Health Service and served in the Hygienic Laboratory, Washington, D. C. In August he obtained leave of absence from the Public Health Service to enter the United States Army as a Second Lieutenant in the Sanitary Corps. He was assigned to duty in the Central Medical Department Laboratory, A. E. F., Dijon, France.

While in the army service, he was appointed to a fellowship in bacteriology at the School of Hygiene and Public Health, Johns Hopkins University and upon his return

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